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Heterogeneous Catalysis Mediated Cofactor NADH Regeneration for Enzymatic Reduction

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Abstract

Enzymatic reduction using oxidoreductases is important in commercial chemical production. This enzymatic action requires a cofactor (e.g. NADH) as hydrogen source that is consumed during reaction and must be regenerated. We present, for the first time, an *in situ* NADH regeneration ($\text{NAD}^+ \rightarrow \text{NADH}$) using a heterogeneous catalyst ($\text{Pt}/\text{Al}_2\text{O}_3$) and H_2 coupled with an enzymatic reduction. This regeneration system can be operated at ambient pressure where NADH yield and turnover frequency (*TOF*) increased with temperature (20-37 °C) and pH (4.0-9.9) delivering full selectivity to enzymatically active NADH. Cofactor regeneration by heterogeneous catalysis represents a cleaner (H^+ as sole by-product) alternative to current enzymatic and homogeneous (electro- and photo-) catalytic methods with the added benefit of facile catalyst separation. The viability of coupling cofactor regeneration with enzymatic (alcohol dehydrogenase, ADH) reaction is established in aldehyde reduction (propanal to propanol) where 100% alcohol yield was achieved. The potential of this hybrid inorganic-enzymatic system is further demonstrated in the continuous (fed-batch) conversion of propanal with catalyst (activity/selectivity) stability for up to 100 h.

Keywords: cofactor NADH regeneration, $\text{Pt}/\text{Al}_2\text{O}_3$, hydrogenation, dehydrogenase, carbonyl reduction

1. Introduction

Enzymatic technologies are extensively employed in the manufacture of chemicals and pharmaceuticals.¹ More than a quarter of commercial enzymes are oxidoreductases² that operate in tandem with a cofactor, typically nicotinamide adenine dinucleotide (NADH) or its phosphorylated form (NADPH). In the enzymatic reaction cycle, NADH serves as hydrogen donor with a resultant oxidation to NAD^+ , as illustrated by pathway (a) in **Figure 1**. Given the high cost of NADH (bulk price per mole: US\$3,000),³ a stoichiometric supply for commercial enzymatic transformations is not economically feasible and regeneration ($\text{NAD}^+ \rightarrow \text{NADH}$, pathway (b) in **Figure 1**) is required. Coupled-enzyme methodologies employ two separate enzymes for substrate \rightarrow product reduction and cofactor regeneration.⁴ Commercial processes use glucose or formate dehydrogenases (GDH or FDH) as regeneration enzymes while phosphite and alcohol dehydrogenases have been applied at laboratory scale.⁴ The generation of significant quantities of water-soluble by-products (196 g gluconic acid per mol NADH regenerated by GDH and 44 g CO_2 per mol NADH regenerated by FDH),^{4a} costly downstream separation and enzyme deactivation are decided drawbacks. Moreover, there is a requirement for base or acid addition to maintain the optimal pH (*ca.* 7.0)⁵ for enzymatic action.⁶ Non-enzymatic NADH regenerations have also been reported employing inorganic reductants (*e.g.* $\text{Na}_2\text{S}_2\text{O}_4$ and NaBH_4),⁷ homogeneous catalysis (*e.g.* Ru, Rh and Ir complexes),⁸ photocatalysis (*e.g.* copolymers and carbon nitride)⁹ and electrocatalysis (including both direct regeneration on the electrode¹⁰ and indirect regeneration using organometallic complexes as hydrogen transfer agents¹¹). Some of these methodologies suffer from low selectivity with irreversible formation of inactive NAD_2 (pathway (c) in **Figure 1**) and/or 1,6-NADH (pathway (d) in **Figure 1**).^{8b,9b,c,10} There are also issues of sustainability associated with the requirement for electron mediators,

photosensitizers, sacrificial organic electron donors and toxic organometallic complexes which necessitate energy-intensive separation stages.^{4a,7,12} Therefore, these non-enzymatic approaches have only been studied at a laboratory scale and not used in industries.

In this work we demonstrate an alternative, novel strategy for NADH regeneration using a heterogeneous (supported metal) catalyst that generates H^+ as *sole by-product*. As supported Pt has shown activity in the hydrogenation of compounds with similar ring structure to NADH (*e.g.* pyridines),¹³ we have investigated a commercial Pt/Al₂O₃ catalyst with H₂ as reductant (see pathway **(b)** in **Figure 1**). This is the first application of supported metal catalyst for cofactor regeneration. We should however flag reports in the literature dealing with (immobilized) enzymatic regeneration using H₂^{12a, 14} and photocatalytic¹⁵ and electrocatalytic¹⁶ regeneration using Pt nanoparticles as photosensitizer and proton carrier, respectively. In this study we couple NADH regeneration by Pt/Al₂O₃ with continuous conversion of propanal to propanol over alcohol dehydrogenase as a model enzymatic redox transformation. We probe the effect of temperature, pH and H₂ pressure in the regeneration step. Our proposed hybrid synthetic-biocatalytic system can serve as a new route for the production of chemicals by NADH dependent enzymes.

2. Experimental

2.1. Materials

β -Nicotinamide adenine dinucleotide hydrate (NAD⁺, $\geq 96.5\%$), β -nicotinamide adenine dinucleotide reduced disodium salt hydrate (NADH, $\geq 94\%$), KH₂PO₄ ($\geq 99\%$), K₂HPO₄ ($\geq 98\%$), DL-6,8-thioctic acid amide (DL-lipoamide, $\geq 99\%$), ethylenediaminetetraacetic acid (EDTA) tetrasodium salt hydrate ($\geq 95\%$), albumin from bovine serum ($\geq 96\%$), diaphorase from *Clostridium kluyveri* (100-UN), alcohol dehydrogenase from *Saccharomyces cerevisiae*, deuterium oxide (D₂O, $\geq 99.9\%$) and Pt/Al₂O₃ (1% w/w) were obtained from Sigma-Aldrich.

Propanal ($\geq 99\%$), propanol ($\geq 99\%$), and ethanol ($\geq 99.8\%$) were supplied by Fisher Scientific. All the chemicals were used as received without further purification. The gases (H_2 , N_2 , and He, BOC) were ultra-high purity ($>99.99\%$).

2.2. Catalyst characterization

Nitrogen adsorption-desorption isotherms (at $-196\text{ }^\circ\text{C}$) were obtained using the commercial automated Micromeritics Gemini VII 2390p system. Specific surface area was obtained from the adsorption isotherms using the standard BET method. Total pore volume ($P/P_0 = 0.95$) and mean pore size was determined by BJH analysis of the desorption isotherms; samples were outgassed at $150\text{ }^\circ\text{C}$ under N_2 for 1 h prior to measurement. Temperature programmed treatment and H_2 chemisorption were recorded on the commercial CHEMBET 3000 (Quantachrome Instrument) equipped with a thermal conductivity detector (TCD) and TPR WinTM software for data acquisition/manipulation. The $\text{Pt}/\text{Al}_2\text{O}_3$ catalyst was loaded into a U-shaped quartz cell (3.76 mm *i.d.*), heated to reaction temperature ($37\text{ }^\circ\text{C}$) under N_2 and subjected to H_2 (BOC, 99.99%) pulse (10-50 μl) titration. The H_2 pulses were continued until the signal area was constant, indicating surface saturation. The as received sample was subjected to thermal treatment in $17\text{ cm}^3\text{ min}^{-1}$ (Brooks mass flow controlled) 5% v/v H_2/N_2 at $5\text{ }^\circ\text{C min}^{-1}$ to $350\text{ }^\circ\text{C}$ for 1 h. The sample was swept with $65\text{ cm}^3\text{ min}^{-1}$ N_2 for 1.5 h, cooled to $37\text{ }^\circ\text{C}$ and subjected to H_2 titration as above. Platinum particle morphology (size and shape) was determined by scanning transmission electron microscopy (STEM, JEOL 2200FS field emission gun-equipped TEM unit), employing Gatan DigitalMicrograph 1.82 for data acquisition/manipulation. By using an annular bright/dark field detector with a minimum collection semi-angle of $\sim 100\text{ mrad}$, the recorded images showed an intensity approximately proportional to $tZ^{1.7-2}$ (sample thickness t , average atomic number Z), facilitating clear contrast between the heavy and lighter element components. Samples for

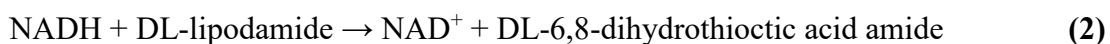
analysis were prepared by dispersion in acetone and deposited on a holey carbon/Cu grid (300 Mesh). The number weighted mean metal particle size (d) was obtained from

$$d = \frac{\sum_i n_i d_i}{\sum_i n_i} \quad (1)$$

where n_i is the number of particles of diameter d_i with $\sum n_i = 440$.

2.3. NADH analysis

NADH concentration was monitored by UV-Vis spectrophotometry (Shimadzu, UVmini-1240) at $\lambda = 340$ nm.^{15,17} In order to discount any contribution to the UV signal from inactive by-products (NAD₂ and 1,6-NADH) an independent validation of NADH yield was required to establish exclusive generation of active NADH. An enzymatic assay using lipoamide dehydrogenase based on Sigma Quality Control Test Procedure EC 1.8.1.4 has been established as an effective means of NADH determination¹⁸ and was employed in this work to identify and quantify NADH concentration. Lipoamide dehydrogenase is specific to NADH and does not act on NAD₂ or 1,6-NADH and only the active NADH is consumed according to



with UV absorbance directly proportional to NADH concentration. The assay was carried out at $T = 25$ °C and pH = 7.0. The following reagents were prepared: **(A)** 0.1 M phosphate buffer solution (pH = 7.0 at 25 °C) from KH₂PO₄ and K₂HPO₄ in deionized water; **(B)** 28 mM DL-6,8-thioctic acid amide solution (DL-Thio, substrate for assay) by dissolving 57.4 mg DL-6,8-thioctic acid amide in 6 cm³ ethanol (non-denatured) and dilution with 4 cm³ phosphate buffer (reagent A); **(C)** 300 mM ethylenediaminetetraacetic acid (EDTA) solution with 2.0% (w/v) albumin (pH = 7.0 at 25°C) by addition of EDTA and albumin to 10 cm³

deionized water and pH adjustment with 5 M HCl; **(D)** product mixture from reaction; **(E)** 1.0 unit cm⁻³ lipoamide dehydrogenase (LDH) solution in cold phosphate buffer (reagent A) to avoid denaturation; **(F)** positive control solution with NADH and NAD⁺ in the phosphate buffer at the same concentration as the product mixture (D). The sample solutions are summarized in **Table 1**. Additional product analysis by ¹H NMR was conducted on a Bruker AVIII (300 MHz) spectrometer at room temperature and reported in ppm with respect to D₂O as internal standard.

2.4. NADH regeneration

NAD⁺ → NADH regeneration was carried out in a Parr[®]5500 compact reactor (with a Parr[®]4848 reactor controller) at 20-60 °C, pH = 4.0-9.9 and over the (H₂) pressure range 1-9 atm. In a typical experiment, Pt/Al₂O₃ (25 mg) and 50 cm³ 0.1 M phosphate buffered solution containing NAD⁺ (1.5 mM) were loaded in the reactor. The system was flushed (three times) with N₂ and the temperature (20-60 °C) allowed to stabilize. Hydrogen gas was then introduced, the system pressurized and stirring (at 900 rpm) engaged (time $t = 0$ for reaction). A non-invasive liquid sampling system via syringe/in-line filters allowed a controlled removal of aliquots from the reactor. NADH yields were calculated from

$$\text{NADH yield (\%)} = \frac{[\text{NADH}]}{[\text{NAD}^+]_0} \times 100 \quad (3)$$

Catalytic regeneration is also quantified in terms of initial rates obtained from a linear regression of temporal NADH concentration profiles¹⁹ with turnover frequency (*TOF*, rate per active site) calculated using Pt dispersion obtained from STEM analysis.

2.5. Propanal conversion with in situ cofactor regeneration

In the combined NADH regeneration and enzymatic conversion of propanal, experiments were carried out at atmospheric pressure using a commercial glass reactor (Ken

Kimble Reactors Ltd.) coupled with a coolant-condenser system operated at $-20\text{ }^{\circ}\text{C}$. At the start of each reaction alcohol dehydrogenase (1 mg, from *Saccharomyces cerevisiae*), NADH (250 mg), Pt/Al₂O₃ (25 mg) and phosphate buffered solution (50 ml, 0.1 M, pH = 8.8) were loaded into the reactor with a N₂ sparge at $30\text{ cm}^3\text{ min}^{-1}$. The temperature was allowed to stabilize (at $20\text{ }^{\circ}\text{C}$), the solution sparged with H₂ ($30\text{ cm}^3\text{ min}^{-1}$) and agitated at 900 rpm with addition of 36 μL (0.5 mmol) propanal; at time $t = 0$, propanal concentration ($[\text{propanal}]_0$) = 10 mM. For the fed-batch process of enzymatic reduction of propanal with *in situ* cofactor regeneration ($T = 20^{\circ}\text{C}$, $P = 1\text{ atm}$, pH = 8.8 and initial NADH = 25 μmol) a syringe pump (KD Scientific) supplied a continuous propanal feed (2 mM in phosphate buffer) at $2.5\text{ cm}^3\text{ h}^{-1}$. The composition of the product mixture was analyzed by gas chromatography (Perkin-Elmer Clarus 580 GC) employing a programmed split injector, flame ionization detector (FID) and DB-WAX (30 m \times 0.25 mm i.d. 0.25 μm film thickness) capillary column (J&W Scientific). Propanol yield was calculated from

$$\text{Propanol yield (\%)} = \frac{[\text{Propanol}]}{[\text{Propanal}]_0} \times 100 \quad (4)$$

Enzymatic activity is also quantified in terms of initial conversion obtained from the time on-stream measurements.¹⁹ Replicated reactions delivered raw data reproducibility to within $\pm 7\%$.

3. Results and discussion

The critical physicochemical characteristics of the as received Pt/Al₂O₃ are given in **Table 2**. The structural measurements (specific surface area = $162\text{ m}^2\text{ g}^{-1}$, pore volume = $0.40\text{ cm}^3\text{ g}^{-1}$ and mean pore size = 7.8 nm) match those reported in the literature.²⁰ As we use H₂ as reductant in cofactor regeneration, a critical catalyst property in this application is H₂ chemisorption capacity. The measured H₂ uptake ($4.1\text{ }\mu\text{mol g}^{-1}$) is comparable to that reported for hydrogenation active alumina supported catalysts (e.g. $4\text{ }\mu\text{mol g}^{-1}$ for Ni/Al₂O₃²¹).

The representative STEM image given in **Figure 2** shows pseudo-spherical Pt particles at the nanoscale (<1-7 nm) with a mean diameter (2.2 nm) that is known to deliver high H₂ uptake.²²

3.1. Cofactor regeneration by heterogeneous catalysis

We first assessed the viability of the proposed catalytic cofactor regeneration route by carrying out batch reduction of NAD⁺ over Pt/Al₂O₃ in H₂. The results are presented in **Figure 3(a)** where the level of reproducibility can be assessed from the error bars generated for five replicated runs. The experimental measurements show an increase in the yield of NADH with time. The results from validation using the EC 1.8.1.4 enzymatic assay (see section 2.3.) are presented in **Figure 3(b)** and demonstrate that the product generated from catalytic treatment exhibited the same assay response to the NADH/NAD⁺ mixture prepared from commercial samples on the basis of UV analysis. The convergence of both sets of results confirms that the catalytic reduction of NAD⁺ is fully selective to NADH. ¹H NMR spectroscopy analysis was used to further demonstrate selective cofactor regeneration. The ¹H NMR spectra for NAD⁺ (**Figure 4(a)**) and NADH (**Figure 4(b)**) present characteristic signals at 9.33 and 6.96 ppm, respectively.²³ The ¹H NMR spectrum of the product mixture after 6 h (**Figure 4(c)**) presents the same signals where the NADH yield determined coincided with UV measurement (see **Figure 3(a)**). This exclusivity in NADH regeneration is an important finding given the number of published studies where the formation of undesired by-products (*e.g.* NAD₂ and 1,6-NADH) has been reported.^{8b,9b,c,10} Dissociative H₂ chemisorption on supported Pt provides surface reactive hydrogen that acts to reduce NAD⁺ to NADH (**Figure 1 pathway (b)**) with the formation of H⁺ as by-product, which is buffered in the reaction mixture. This is in contrast to conventional enzymatic regenerations where the formation of

water-soluble by-products requires labor-intensive downstream separation and purification steps.

Temperature, pH and pressure are critical reaction parameters that impact on enzymatic performance.³ The influence of each variable in our catalytic regeneration process is shown in **Figure 5**. NADH yield **(i)** and specific activity in terms of turnover frequency (*TOF*) **(ii)** were temperature dependent (**Figure 5(a)**). Over a typical temperature range for enzymatic reactions (20 to 37 °C),²⁴ temporal NADH yield showed an increase with temperature. At a higher reaction temperature (60 °C) a maximum yield was observed after 1.5 h with a decrease thereafter that can be linked to reduction of the nicotinamide ring. A temperature maximum (40 °C) in NAD⁺ conversion during enzymatic regeneration using a redox mediator (flavin adenine dinucleotide) has been reported and attributed to deactivation of FDH by the electron mediator.²⁵ In order to account for the response at 60 °C we monitored catalytic regeneration at extended reaction times (up to 24 h). We should stress that regeneration procedures reported in the literature have been limited to shorter duration, from 2^{9b,26} to 12 h¹⁵. Analysis of the reaction mixture after 6 h by ¹H NMR (**Figure 4(d)**) showed no signal due to NAD⁺ (δ = 9.33 ppm) consistent with increased activity at higher temperatures (**Figure 5(aii)**) and the appearance of signals at 1-2 ppm. The intensity of these signals increased after 24 h with no detectable NADH (δ = 6.96 ppm) (**Figure 4(e)**). Peaks at 1-2 ppm are characteristic of saturated hydrocarbons²⁷ and can be attributed to product(s) resulting from hydrogenation of the nicotinamide ring. Our results confirm that NADH regeneration is temperature dependent with undesired ring hydrogenation at 60 °C. Subsequent tests were conducted at $T \leq 37$ °C to avoid non-selective cofactor transformation.

Our regeneration system also showed a pH dependence (**Figure 5(b)**) where an increase in pH from 4.0 to 9.9 resulted in substantial enhancement in NADH yield with an associated

increase in *TOF*. A basic environment serves to neutralize and remove H^+ released in the regeneration step (**Figure 1(b)**) shifting the reaction in favor of NADH formation. A similar pH effect (from 6.0 to 10.0) has been reported for photocatalytic cofactor regeneration.^{9b} We should note that Ir complex (homogeneous) catalyzed NADH regeneration at pH = 4–10 with a maximum at pH = 6.5.¹⁷ Rate inhibition was shown at pH > 8. This is in contrast to our system where higher pH favors higher conversion. An increase in H_2 pressure (1-9 atm) served to increase NADH yield and *TOF* (**Figure 5(c)**). This can be ascribed to enhanced H_2 availability in the aqueous medium. The higher *TOF* on raising system pressure from 1 to 5 atm matches the increase in hydrogen solubility (0.0138 vs. 0.0875 $cm^3\ g^{-1}$).²⁸ Further pressure elevation (to 9 atm, H_2 solubility = 0.154 $cm^3\ g^{-1}$) did not result in a proportional increase in rate. An overriding practical consideration for cofactor regeneration when coupled with enzymatic reaction is operation at ambient pressure.^{4a} The as received catalyst delivered a *TOF* (4 h^{-1}) under ambient conditions and was increased to 13 h^{-1} (**Figure 5(d)**) following thermal treatment of the catalyst (to 350 °C) in H_2 . The treated Pt/ Al_2O_3 exhibited equivalent structural characteristics (see **Table 2**) but with a significantly higher level (5-fold) of H_2 chemisorption which can be linked to further reduction of surface Pt to the metallic form with greater H_2 uptake capacity and a consequent increased NADH regeneration rate. In summary, we have established the viability of NADH regeneration using Pt/ Al_2O_3 with H_2 as reductant under conditions that are suitable for enzymatic reaction. The regeneration rate (12 $\mu mol\ min^{-1}\ mg^{-1}$) over Pt/ Al_2O_3 is six times higher than NAD^+ -reducing hydrogenase (2 $\mu mol\ min^{-1}\ mg^{-1}$)²⁹ and comparable with values reported for homogeneous organometallic catalysts (13 h^{-1} in this work relative to 10 h^{-1} with formate,²⁶ 18 h^{-1} using ethanol³⁰ and 36 h^{-1} using H_2 ¹⁷) but those systems exhibit incompatibilities between the organometallics and NADH dependent enzymes that result in enzyme deactivation.³¹ Regeneration using immobilized

organometallic catalyst $[\text{Cp}^*\text{Rh}(\text{bpy})(\text{H}_2\text{O})]^{2+}$ has also been reported but showed a maximum *TOF* of 2.5 h^{-1} at 30°C ,³² five times lower than our system.

3.2. Batch enzymatic propanal reduction with *in situ* cofactor regeneration

Practical viability of cofactor regeneration requires applicability *in situ* in tandem with a biocatalytic process. Taking this as our goal, we have developed a “one-pot” system that combines NADH regeneration by heterogeneous catalysis with enzymatic (alcohol dehydrogenase) conversion of propanal to propanol as model reaction. The results of the combined cofactor regeneration and propanal transformation are presented in **Table 3** and **Figure 6**. As a blank test (control experiment), propanal conversion (in H_2) over $\text{Pt}/\text{Al}_2\text{O}_3$ delivered a rate four orders of magnitude lower than that achieved with alcohol dehydrogenase (**Table 3**, rows **1** and **2**). This result demonstrates that propanal transformation is governed by the enzyme and is consistent with the equivalent propanol production rate obtained in the one-pot system with or without $\text{Pt}/\text{Al}_2\text{O}_3$ (**Table 3**, rows **2** and **3**). The production of propanol is limited by the available NADH concentration and without $\text{Pt}/\text{Al}_2\text{O}_3$ addition propanol yield reached an upper limit of 70%. Cofactor regeneration by $\text{Pt}/\text{Al}_2\text{O}_3$ extended alcohol production beyond the initial NADH/propanal stoichiometry to reach full conversion (100% yield, **Figure 6**). The rate of propanol production over the combined $\text{Pt}/\text{Al}_2\text{O}_3$ and cofactor ($0.100 \mu\text{mol min}^{-1}$) at the point the two profiles deviate ($t > 5 \text{ min}$) matched the rate of NAD^+ reduction by $\text{Pt}/\text{Al}_2\text{O}_3$ ($0.095 \mu\text{mol min}^{-1}$). The hydrogenation performance of the coupled system is ultimately controlled by the rate of NADH regeneration. The heterogeneous catalyst ($\text{Pt}/\text{Al}_2\text{O}_3$) plays an exclusive role in regenerating the NADH cofactor which regulates enzymatic production of the alcohol.

3.3. Continuous enzymatic propanal reduction with *in situ* cofactor regeneration in a fed-batch setup

In order to realize the full potential of this *in situ* cofactor regeneration strategy we investigated the feasibility of fed-batch propanol production using a fixed starting amount of NADH with continuous propanal supply and cofactor regeneration by Pt/Al₂O₃. Continuous processing has been highlighted as crucial for the sustainable manufacture of chemicals and is now a primary area for process development³³ because continuous operation overcomes the drawback of unproductive “down time” between batches, which is an imperative for the application of large scale biocatalysis in industry.³⁴ Without cofactor regeneration propanol production is limited by the initial NADH concentration (**Figure 7**). Propanol production with a continuous feed was achieved through the combined catalytic action of Pt/Al₂O₃ and alcohol dehydrogenase (**Figure 7a**) where a constant level of propanol production was maintained in operation for up to 100 h. The overall turnover number for NADH is 7, suggesting that further improvement would be beneficial. In another experiment, the reaction was started *without* NADH and the supply of cofactor was entirely dependent on regeneration from NAD⁺ (**Figure 7b**). The formation of propanol has been increasing progressively while the control without Pt/Al₂O₃ showed no propanol produced. The results once again proved that the cofactor regeneration using heterogeneous catalysis was successful and the regenerated NADH was active.

As a proof of concept, this study demonstrates mutual compatibility and stability for the synthetic and enzymatic catalytic components. We can note recent work by Roche *et al.*³⁵ that examined NADH regeneration using immobilized FDH in a continuously supplied reactor for the enzymatic reduction of pyruvate to L-lactate. Starting with NAD⁺ the immobilized FDH exhibited a much lower activity (2.0×10^{-5} - 5.5×10^{-5} $\mu\text{mol min}^{-1}$) than achieved in this

study with a significant loss (by 65%) of activity over 20 days. Liu *et al.*³⁶ have reported the coupling of photocatalytic regeneration (using carbon nitride prepared *via* fluoride etching) with enzymatic reduction of formaldehyde to methanol at a small (3 cm³) scale. This system required [Cp*Rh(bpy)H₂O]²⁺, triethanolamine and a high photocatalyst loading (3 mg) with alcohol dehydrogenase (0.15 mg ml⁻¹) to achieve a rate of 0.21 μmol min⁻¹ mg_{enzyme}⁻¹.

Our encouraging results from the fed-batch operation suggest that this combined enzymatic-heterogeneous catalytic system can be developed further for wide range of NADH-dependent enzymatic syntheses at a larger scale. This can potentially close the sustainability gap in cofactor utilization in enzymatic reduction processes. Further research has been set out to focus on the application of this coupled system to the production of chiral alcohols, exploiting the full potential of enzymatic catalysis.

4. Conclusions

This study has established for the first time the viability of heterogeneous catalytic (using Pt/Al₂O₃ and H₂) enzyme cofactor (NADH) regeneration. This procedure exhibits advantages in terms of full selectivity, stability, waste minimization (H⁺ as sole by-product), facile catalyst separation and process sustainability (circumventing toxic metal complexes, electron mediators, sacrificial electron donors and photosensitizers). Cofactor regeneration using Pt/Al₂O₃ can be operated under ambient conditions and a wide range of pH (4–9.9) which facilitate coupling with enzymatic reaction. We have demonstrated the feasibility of incorporating *in situ* regeneration with an enzymatic process (reduction of propanal to propanol by alcohol dehydrogenase) in continuous (fed-batch) operation. Our methodology delivers effective NADH utilization with potential cost savings and paves new alternative pathways for cofactor regeneration.

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Table 1: Amount of reagent (in cm^3) required for the preparation of the lipoamide dehydrogenase assay.

Reagent	Blank	A_0	A	A'_0	A'
A (buffer)	2.0	1.0	0	1.0	0
B (DL-Thio)	0.2	0.2	0.2	0.2	0.2
C (EDTA)	0.1	0.1	0.1	0.1	0.1
D (product)	0	1.0	1.0	0	0
E (LDH)	0	0	1.0	0	1.0
F (positive control)	0	0	0	1.0	1.0

Table 2: Physicochemical characteristics of Pt/Al₂O₃.

	Pt/Al ₂ O ₃ (as received)	Pt/Al ₂ O ₃ (treated in H ₂ at 350 °C)
Specific surface area (m ² g ⁻¹)	162	175
Pore volume (cm ³ g ⁻¹)	0.40	0.43
Mean pore size (nm)	7.8	8.0
<i>d</i> (nm)	2.2	3.4
H ₂ chemisorption (μmol g ⁻¹)	4.1	21.5

Table 3: Rate and yield of propanol from propanal reduction with an enzyme/NADH/Pt/Al₂O₃ component matrix. Row **1**: control without enzyme; row **2**: enzymatic production of propanol without cofactor regeneration; row **3**: reaction with cofactor regeneration. *Reaction conditions:* $T = 20\text{ }^{\circ}\text{C}$, $\text{pH} = 8.8$, $P = 1\text{ atm}$.

	Propanal (36 μL)	Enzyme (1 mg)	NADH (250 mg)	Pt/Al ₂ O ₃ (25 mg)	Propanol production rate ($\mu\text{mol}_{\text{propanol}}\text{ min}^{-1}\text{ mg}_{\text{enzyme}}^{-1}$)	Propanol yield (%)
1	✓			✓	0.004 ^a	0
2	✓	✓	✓		138	70
3	✓	✓	✓	✓	143	100

^a units: $\mu\text{mol}_{\text{propanol}}\text{ min}^{-1}\text{ mg}_{\text{Pt/Al}_2\text{O}_3}^{-1}$

Figure 1

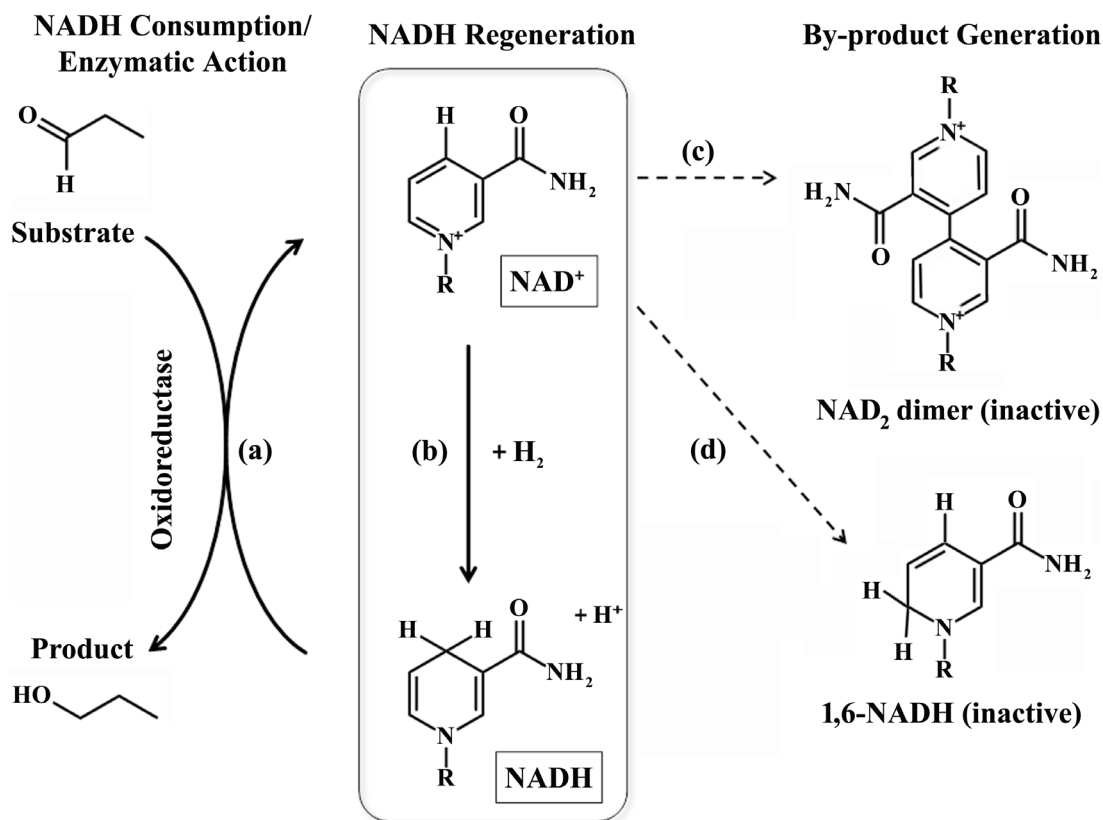


Figure 1: Schematic representation of (oxidoreductive) enzymatic reaction (propanal → propanol) using NADH as a cofactor and possible products obtained from NADH regeneration: **(a)** NADH consumption in oxidoreductase biotransformation; **(b)** target pathway for NADH regeneration; formation of undesired (dashed arrows) inactive **(c)** NAD₂ dimer and **(d)** 1,6-NADH. *Note:* R indicates adenosine diphosphoribose.

Figure 2

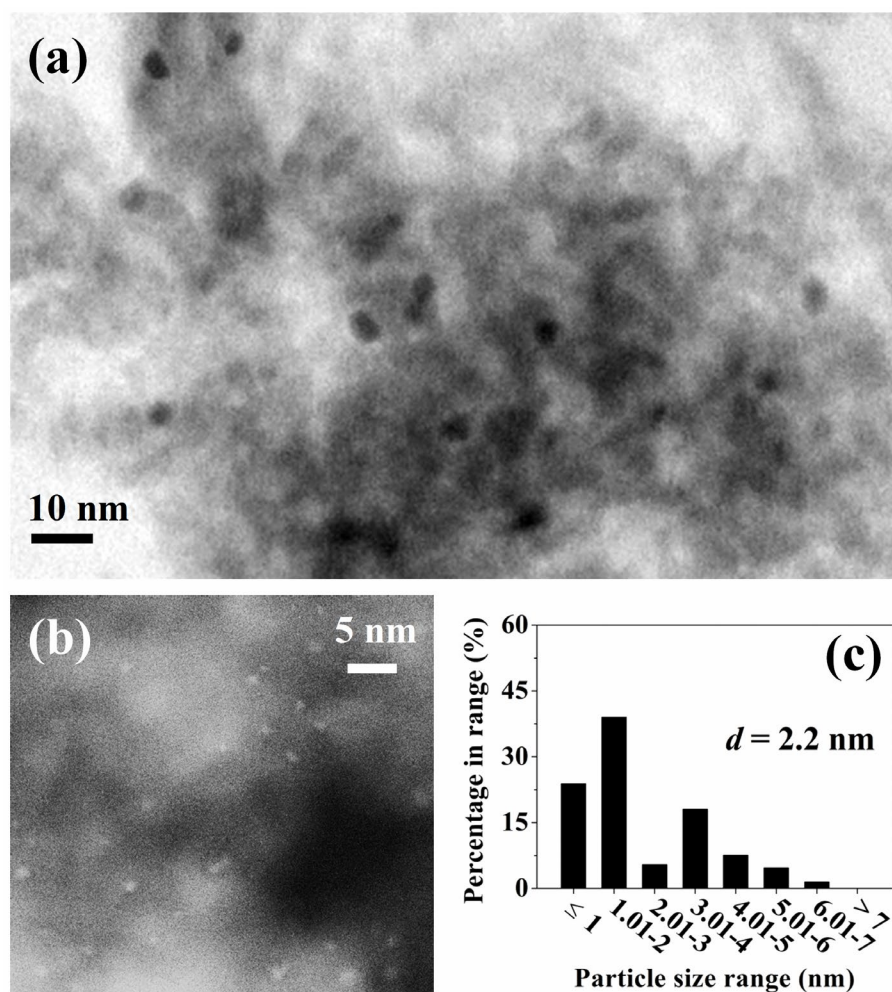


Figure 2: Representative STEM images of as received Pt/Al₂O₃ in bright field (a) and dark field (b) with associated Pt particle size distribution (c).

Figure 3

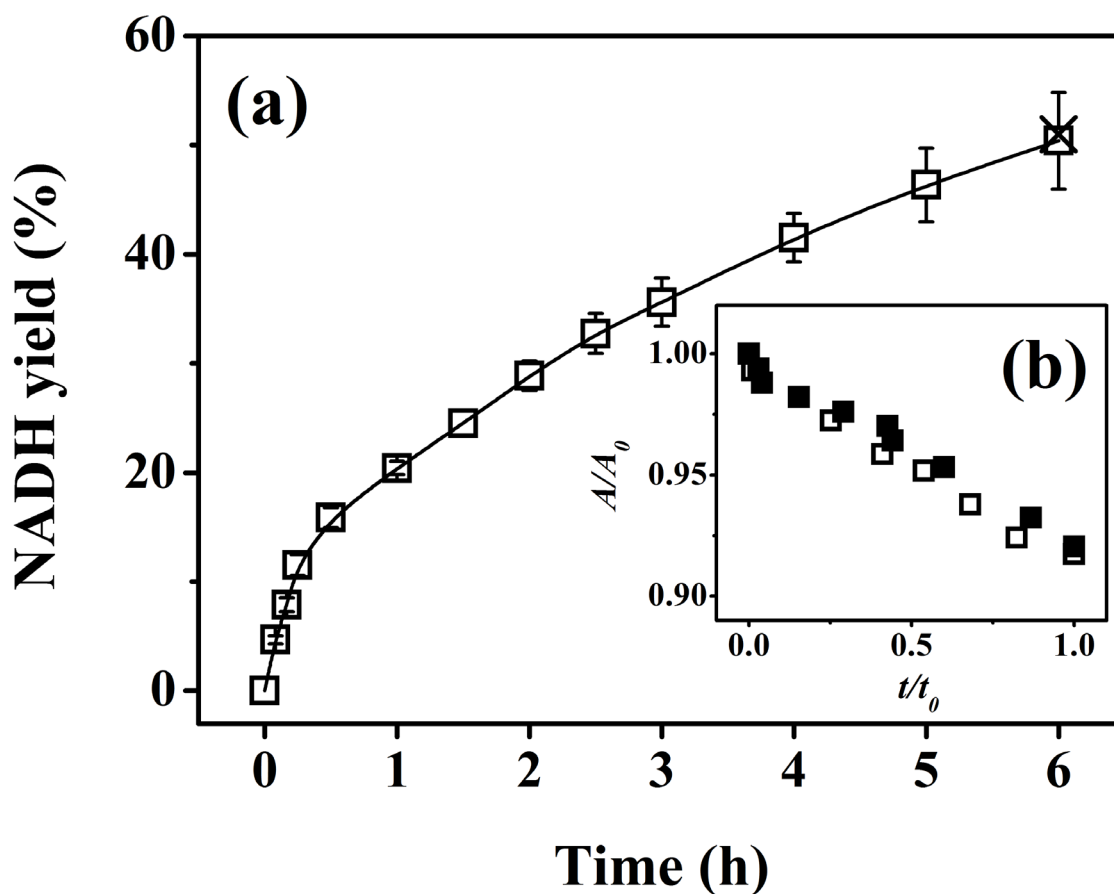


Figure 3: Pt/Al₂O₃ catalyzed NAD⁺ → NADH regeneration. **(a)** Variation of NADH yield as a function of time (□, Reaction conditions: $T = 37\text{ }^{\circ}\text{C}$, $P = 9\text{ atm}$, $\text{pH} = 7$, $[\text{NAD}^+]_0 = 1.5\text{ mM}$) with NADH yield determined by ¹H NMR (×); **(b)** NADH yield validation using enzymatic assay (EC 1.8.1.4): time dependence of normalized absorbance (A/A_0) of NADH produced experimentally (□) and from a prepared mixture using commercial NADH and NAD⁺ (■, see Experimental Section): A_0 is the absorbance recorded before the enzymatic assay; A is the absorbance recorded after reaction is initiated; t_0 is the total run time of the enzymatic assay; t is the time after initiating the reaction (see Experimental Section).

Figure 4

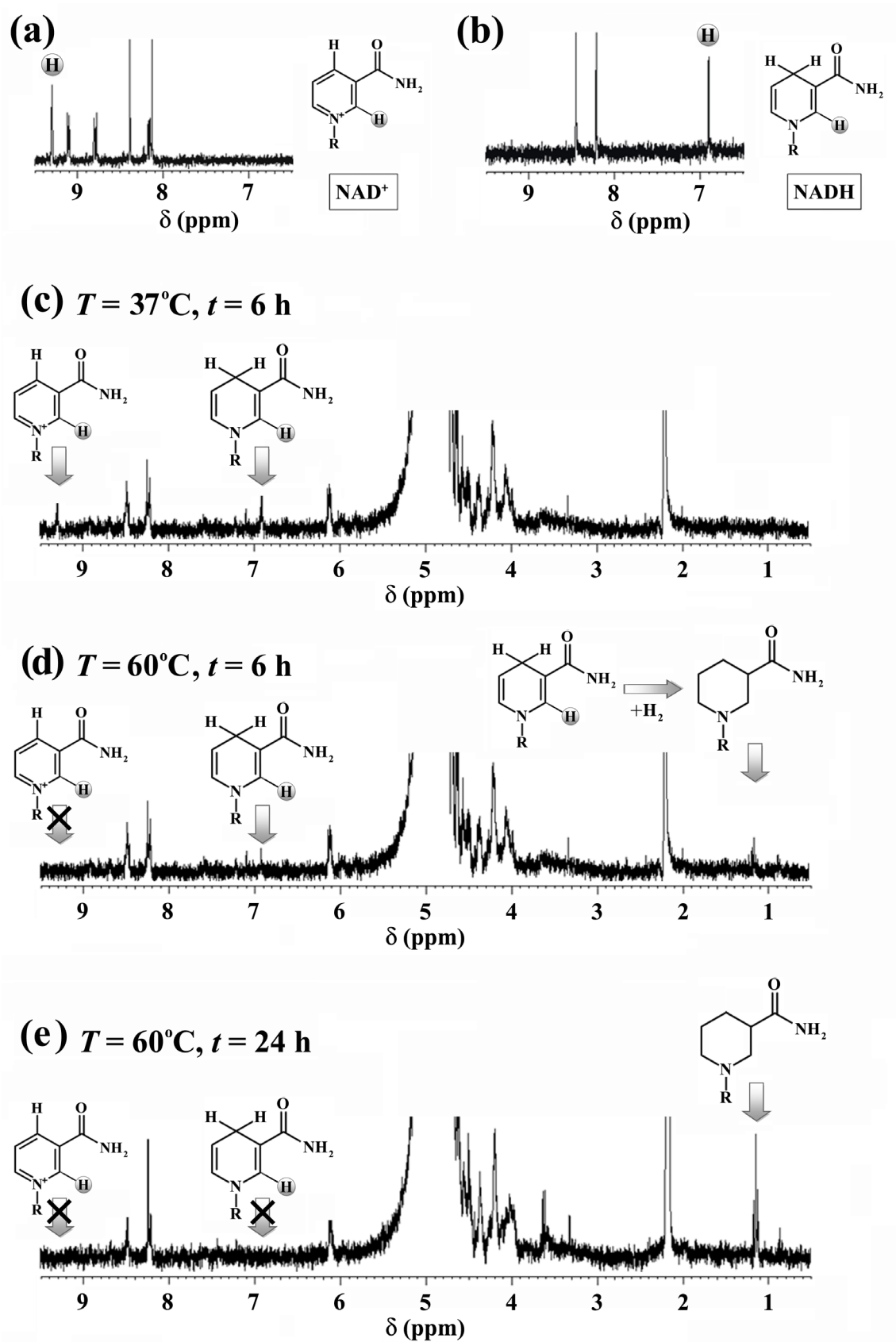


Figure 4: ^1H NMR spectra. **(a)** 1.5 mM NAD^+ in 100 mM phosphate buffer (pH = 7.0); **(b)** 1.5 mM NADH in 100 mM phosphate buffer (pH = 7.0); reaction samples from Pt/ Al_2O_3 promoted NAD^+ reduction ($P = 9$ atm and pH = 7.0) at **(c)** 37 °C after 6 h and at 60 °C after **(d)** 6 h and **(e)** 24 h.

Figure 5

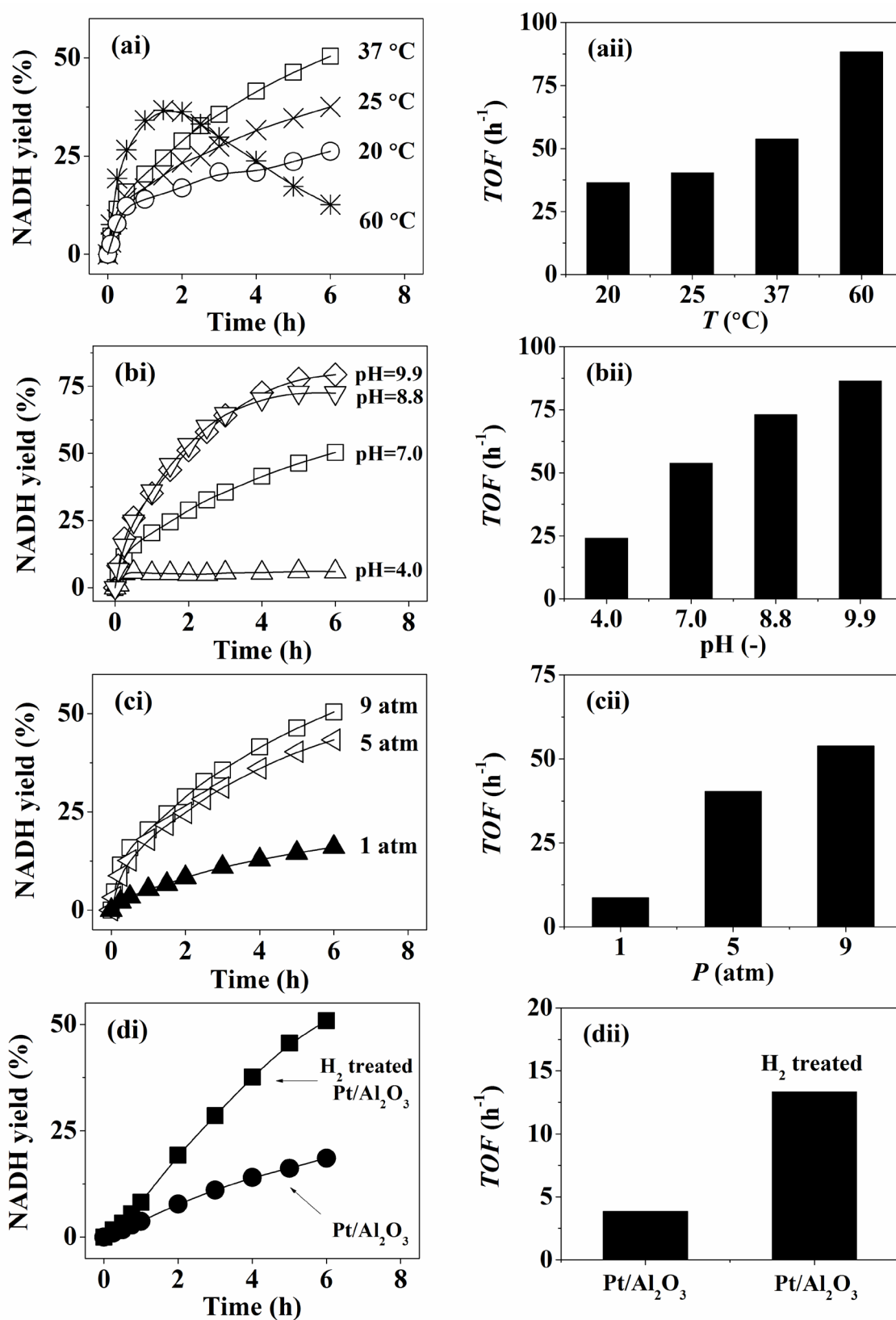


Figure 5: (i) Temporal variation of NADH yield and (ii) turnover frequency (*TOF*) as a function of: (a) temperature (20 °C (○) 25 °C (×), 37 °C (□) and 60 °C (*)) at $P = 9$ atm, $pH = 7.0$); (b) pH

(4.0 (Δ), 7.0 (\square), 8.8 (∇) and 9.9 (\diamond) at $T = 37\text{ }^{\circ}\text{C}$, $P = 9\text{ atm}$); **(c)** pressure, (1 atm (\blacktriangle), 5 atm (\triangleleft) and 9 atm (\square) at $T = 37\text{ }^{\circ}\text{C}$, pH = 7.0); **(d)** H_2 treatment (Pt/ Al_2O_3 as received (\bullet) and H_2 treated Pt/ Al_2O_3 (\blacksquare) at $T = 20\text{ }^{\circ}\text{C}$, $P = 1\text{ atm}$, pH = 8.8).

Figure 6

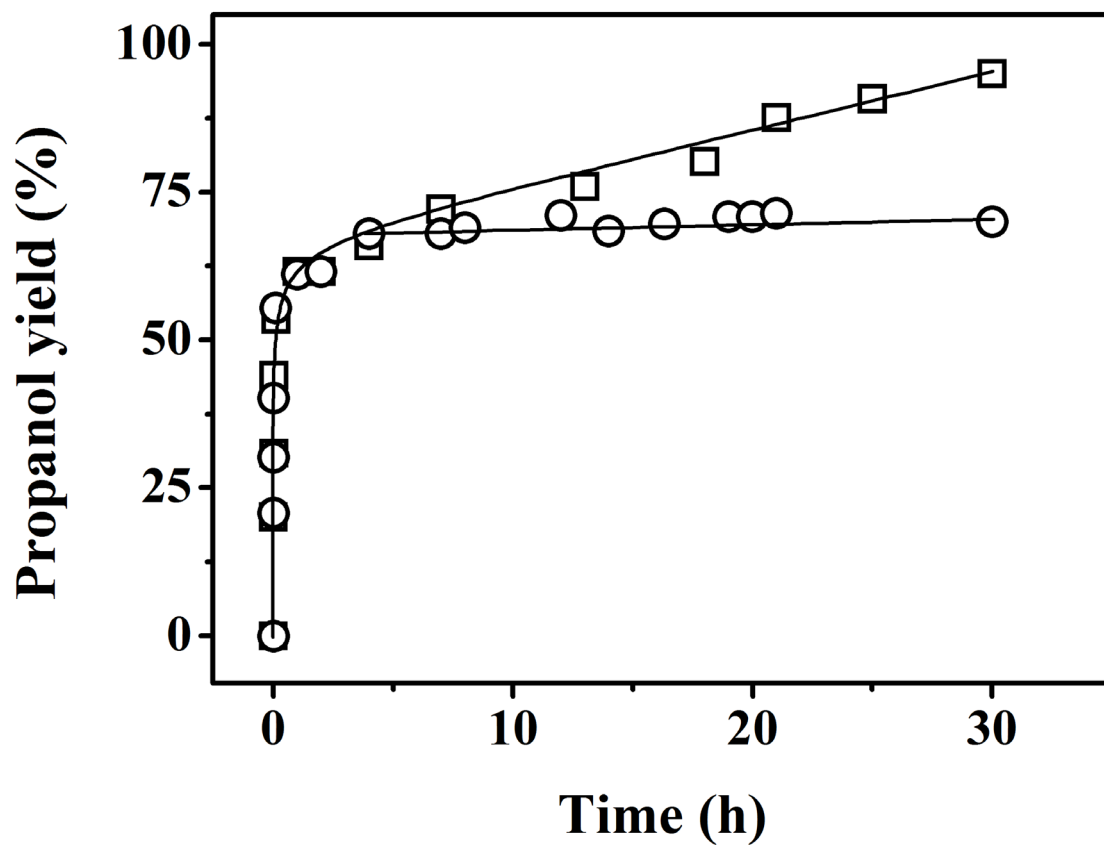


Figure 6: Batch enzymatic reduction of propanal to propanol coupled with *in situ* NADH regeneration by Pt/Al₂O₃. Temporal propanol yield over enzyme + NADH (O) and enzyme + NADH + H₂ + Pt/Al₂O₃ (□).

Figure 7

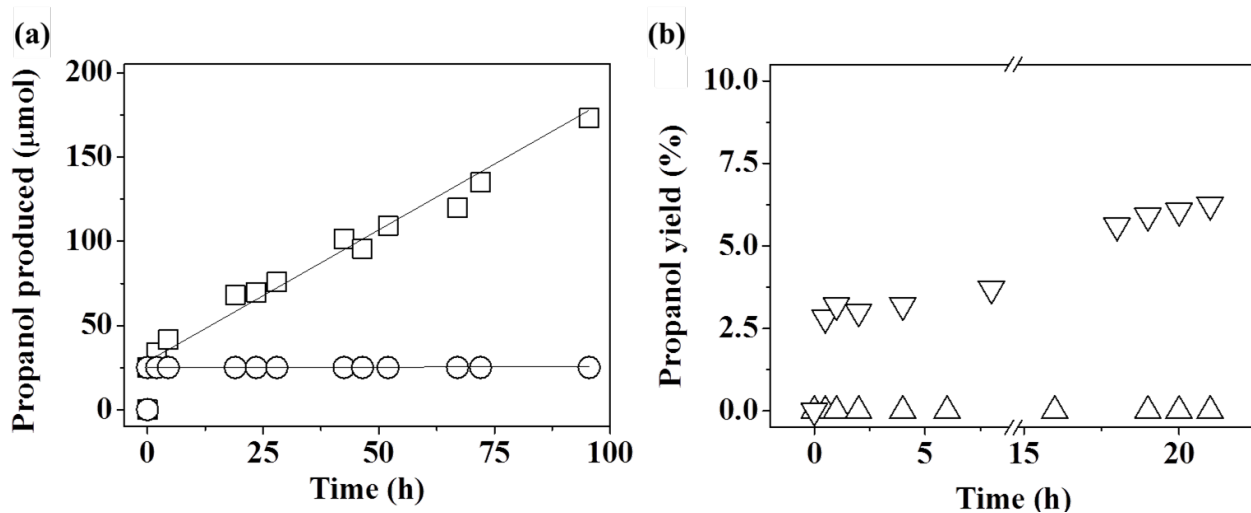
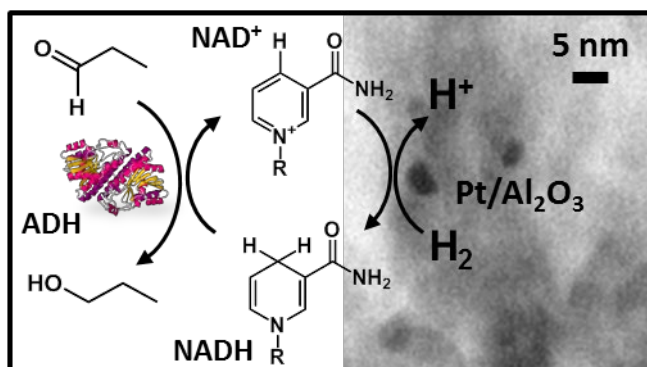


Figure 7: Continuous enzymatic reduction of propanal to propanol coupled with *in situ* NADH regeneration by $\text{Pt/Al}_2\text{O}_3$ in a fed-batch system. (a) Propanol production as a function of time with (\square) and without (\circ) *in situ* NADH regeneration. (b) Temporal variation of propanol yield over alcohol dehydrogenase + NAD^+ (\triangle) and alcohol dehydrogenase + NAD^+ + H_2 + $\text{Pt/Al}_2\text{O}_3$ (∇). Initial NAD^+ : 330 mg. *Reaction conditions:* $T = 20\text{ }^\circ\text{C}$, $P = 1\text{ atm}$, $\text{pH} = 8.8$ and propanal feed (concentration = 2 mM in buffer) rate = $2.5\text{ cm}^3\text{ h}^{-1}$.

TOC figure:



A hybrid inorganic-enzyme catalytic system was developed to reduce propanal to propanol with an *in situ* cofactor regeneration using Pt/Al₂O₃ as the catalyst.